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14. ABSTRACT

Heterogeneity, variable and often unpredictable clinical course are fundamental challenges in management of patients with prostate cancer. To make rapid advances in understanding of disease mechanism that can be translated to clinical care in short order, there is an immediate need for innovative in vivo disease models that accurately recapitulate human disease at cellular level. We propose to develop an innovative and hitherto not attempted in vivo prostate cancer model that will delineate the exact cell of origin through different stages of prostate cancer development and progression. We propose to study possible cell(s) of origin for prostate cancer by combinatorial expression of florescent magenta, cyan and yellow primary color proteins in prostate at development in mice. The study includes (1) Construction of "Prorainbow" plasmid with fluorescent proteins (XFPs) under control by prostate epithelial and basal cell-specific promoters. (2)Establish mouse line with the resulting "Prorainbow" construct and generation of transgenic mice by crossing with Cre mice. (3) Study the transgenic Prorainbow mice under normal and oncogenic conditions. The study is expected to produce in vivo animal of prostate cancer with unique capabilities.

15. SUBJECT TERMS
Prostate cancer, in vivo model, Prorainbow, tumor development

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Annual Report

Title: Tracking Origins of Prostate Cancer – an Innovative *in vivo* Modeling

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Introduction

Prostate cancer is the second most frequently diagnosed cancer of men and the fifth most common cancer overall. The cancer cells may metastasize from the prostate to other parts of the body, particularly the bones and lymph nodes. About 20% of patients undergoing radical prostatectomy develop metastasis beyond 5 years, suggesting metastasis is an early event and removal of primary tumor does not significantly decrease the rate of metastasis. Thus, understanding the role of the genetic changes leading to origination and development of primary tumor and metastasis would provide for a targeting strategy to clinical therapy. The goal of this project is to study the origin of cancer cells within the prostate. Since development of human prostate cancer proceeds through a serious of defined states, we would utilize a newly developed fluorescent protein labeling technique, Brainbow, which has been used to study the nervous system development in Brain[1]. Similar to the 'Brainbow' concept we propose 'Prorainbow' modeling to track prostate cell proliferation and differentiation by labeling individual early prostate precursor cell a unique color. In case of a tumor or metastasis, we can track down the ancestor normal cell by matching to the tumor cell color. We can then track these color distributions and pattern changes with time course, which will build up a dynamic vision of prostate cancer progression. Also, we want to examine functions of Protein Kinase D1 (PKD1) and Phosphatase and Tensin homolog (Pten) in conditional knockout mice in the development of cancer formation and metastasis in the prostate. Successful development of florescent labeled *in vivo* animal model will be unique in the field of prostate cancer research and provide much needed advance to understand progression of prostate cancer.

We proposed to testify the stated hypothesis with following aims:

1) Construction of 'Prorainbow' plasmid with fluorescent proteins (XFPs) under control by prostate epithelial and basal cell-specific promoters.
2) Establish mouse line with the resulting 'Prorainbow' construct and generation of transgenic mice by crossing with Cre mice.
3) Study the transgenic Prorainbow mice under normal and oncogenic conditions.

Body

Aim (1) Construction of 'Prorainbow' plasmid with fluorescent proteins (XFPs) under control by prostate epithelial and basal cell-specific promoters.

*Task I***.** Generation of *Probasin* promoter controlled XFP.

Probasin is a prostate specific and androgen-regulated protein, which can be used as a marker of prostate differentiation. The rat probasin promoter (ARR2PB), which is 455 bp in size, has been successfully cloned and used in transgenic mice to target high-level, prostate-specific expression of down-stream transgenes[2], and the expression regulated by Cre recombinase is in both basal and luminal epithelial cells. We amplified the PB promoter by PCR from pPr-luc (Addgene #8392) and specific primers (PB-F: 5'-AGTCATT AATAAGCTTCCACAAGTGCATTTAGCCTCTCC-3'; PB-R: 5'- AGTCGCTAGCCTGTAGGTATCTGGACCT CACTGAC-3') (Figure1), and successfully ligated the promoter into Brainbow 1.0L vector to replace the original CMV promoter (Figure1). We designed primers (5' AseI check: 5'-AGCCTATGGAAAAACGCCAG-3'; 3' NheI check: 5'-ATCAAAGAGTTCATGCGCTT-3') for further sequencing to make sure the promoter sequence is intact and of no point mutations.

*Task II***.** Generation of *Cytokeratin 5* promoter controlled XFP.

Cytokeratin (CRK) 5 is expressed specifically in basal layer of all stratified squamous epithelia[3-5]. After the differentiation, CRK5 expression would be gradually lost[6], which makes CRK5 promoter an ideal candidate for

direction of XFP expression. We amplified the CRK5 promoter (907 bp) by PCR using human genomic DNA as template and specific primers (CRK5-F: 5'- AGTCATTAATGATCCCCGGGTTTCCTAAACC-3'; CRK5-R: 5'-AGTCTCTAGA GGCTTGTTCCTGGTGGAGCAAGAGAAC-3') (Figure1), and successfully ligated the promoter into Brainbow 1.0L vector to replace the original CMV promoter (Figure1). We designed primers (5' AseI check: 5'-AGCCTA TGGAAAAACGCCAG-3'; 3' NheI check: 5'-ATCAAAGAGTTCATGCGCTT-3') for further sequencing to make sure the promoter sequence is intact and of no point mutations.

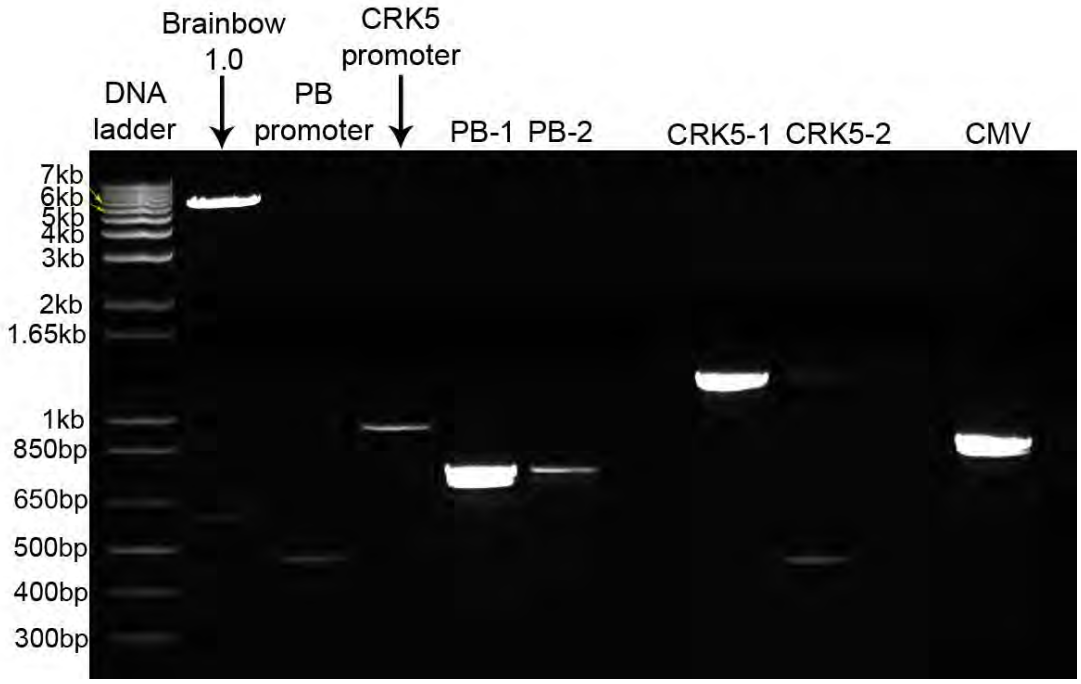

Figure 1 Subcloned Probasin promoter and Cytokeratin 5 promoter into the Brainbow vector. Brainbow 1.0, the Brainbow 1.0L plasmid digested by AseI and NheI. The upper band is the backbone (6.28kb), and the lower band is the CMV promoter (574bp). PB promoter, the probasin promoter amplified by PCR using primers PB-F and PB-R (expected size 475bp). CRK5 promoter, the Cytokeratin 5 promoter amplified by PCR using primers CRK5-F and CRK5-R (expected size 927bp). PB-1, PB-2, the probasin promoter and extension area amplified by PCR (templates are PB-Brainbow candidate clone 1 and 2, and the primers are 5' AseI check and 3' NheI check) (expected size 740bp). CRK5-1, CRK5-2, the cytokeratin 5 promoter and extension area amplified by PCR (templates are CRK5-Brainbow candidate clone 1 and 2, and the primers are 5' AseI check and 3' NheI check) (expected size 1212bp). CMV (control), the CMV promoter and extension area amplified by PCR (template is Brainbow 1.0L, and the primers are 5'AseI check and 3' NheI check) (expected size 865bp).

Aim (2) Establish mouse line with the resulting 'Prorainbow' construct and generation of transgenic mice by crossing with Cre mice.

Task I. Obtain institutional approval for animal study.

Since the PI and the whole lab transferred to Wake Forest University (WFU), we submitted a new animal protocol at WFU to get the permission of carrying out all the experiments proposed. The animal protocol is approved by Institutional Animal Care and Use Committee (IACUC) at WFU (Protocol # A11-097). Later we submitted an animal use appendix to USAMRMC Animal Care and Use Review Office (ACURO) and got the granted approval from ACURO (Appendix1).

Task II. Generation of Prorainbow construct expressing transgenic mice (n=3~5).

The pronuclear injection of the Prorainbow fragment into C57 BL/6 F1 mouse fertilized eggs and the placement of the embryos into pseudo-pregnant females will be performed at Transgenic Mouse Core at Wake Forest University School of Medicine. Potential founder animals will be genotyped and at least three independent lines will be obtained for each construct.

Timeframe: 6-30 months

We plan to work on this task in the following 24 months.

Task III. Cross-breeding of Cre mice with Prorainbow transgenic mice.
Three different crossings will be set up to validate the Prorainbow system and to study normal prostate development.

1) RoSA-CreER X PB-XFP
2) PB-Cre4 x CRK5-XFP
3) PB-Cre4 x CMV-XFP

The offspring from first mating are expected to show a default red color in whole prostate (both basal and luminal epithelial cells). The offspring from second mating are expected to show a default red color in prostate basal cells, and prostate stem cells which reside in basal layers would carry unique color spectra. Also, the fluorescence will decrease in daughter cells after each cell cycle, given the fact that CRK5 promoter activity diminishes with progressive differentiation. The offspring from third mating are expected to have a whole body red color with unique color spectra in prostate only.

Timeframe: 12-30 months

Task IV. Cross-breeding of Prorainbow transgenic mice for cancer research.

We will generate PKD1 and Pten double knockout (specifically knocked out in prostate) mice by crossing PB-Cre4, $PKD1^{lox/lox}$ and $Pten^{lox/lox}$ mice, and check whether the double knockout animals would have increased possibility to initiate primary tumor or start metastasis.

Timeframe: 18-30 months
Deliverables: PTEN or PKD1 knock-out mice expressing Prorainbow constructs in prostate.

Aim (3) Study the transgenic Prorainbow mice under normal and oncogenic conditions.

Task I. Evaluate combinatorial expression of XFP in prostate of Prorainbow mice.

We will study the anatomical distribution of color spectra in the prostate to see whether the same lineage cells localize focally or diffusely. Also, we will study the number of different spectra can be seen in a prostate gland to check the minimal number of stem cells that is required for gland formation.

Time Frame: 18-36 months

Task II. Study of Prorainbow and PTEN or PKD1 knock-out mice hybrids.

We will detect metastasis in PKD1 Pten double knockout mice by flow cytometry analysis of tumor cells in circulation system (which are XFP positive under the expression control of PB promoter, and are of prostate origin). Pten knockout mice will be used as control. In case the tumor cells are detected in circulation, the mouse will be sacrificed and the organs known for prostate cancer metastasis (e.g. bone marrow, lymphoid, lung, liver) will be examined for XFP positive cells. If metastatic cells are found, we will establish whether these cells have the same color spectrum(monoclonal) or diverse color spectra (polyclonal). We can also track back the prostate progenitors of the metastatic cells by matching the color spectra.

Timeframe: 18-36 months
Deliverables: Data analysis based on expression pattern of Prorainbow during prostate development, cancer development and progression.

Key research accomplishments

We have amplified the PB promoter and CRK5 promoter and constructed PB-rainbow and CRK5-rainbow constructs. As soon as the sequence is confirmed by sequencing, we will send them to Transgenic Core and have the transgenic mice produced. We also have got the approval from ACURO to carry out our animal experiments, and we started the animal breeding and crossing for future experiments.

Reportable outcomes

none

Conclusion

Probasin promoter and cytokeratin 5 promoters could be successfully subcloned into the Brainbow vector to replace the CMV promoter.

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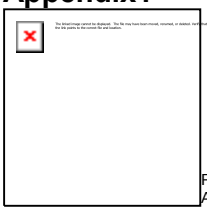
Appendix1



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MD 21702-5012

REPLY TO
ATTENTION OF

July 05, 2011

Director, Office of Research Protections
Animal Care and Use Review Office

Subject: Review of USAMRMC Proposal Number PC094003, Award Number W81XWH-10-1-0709 entitled, "Tracking Origins of Prostate Cancer: An Innovative In Vivo Modeling"

Principal Investigator Kethandapatti Balaji
Wake Forest University Health Sciences (WFUHS)
Winston-Salem, NC

Dear Dr. Balaji:

Reference: (a) DOD Instruction 3216.01, "Use of Animals in DOD Programs"
(b) US Army Regulation 40-33, "The Care and Use of Laboratory Animals in DOD Programs"
(c) Animal Welfare Regulations (CFR Title 9, Chapter 1, Subchapter A, Parts 1-3)

In accordance with the above references, protocol PC094003 entitled, "Tracking Origins of Prostate Cancer-An Innovative In Vivo Model," IACUC protocol number A11-097 is approved by the USAMRMC Animal Care and Use Review Office (ACURO) for the use of mice and will remain so until its modification, expiration or cancellation. This protocol was approved by the Wake Forest University IACUC.

When updates or changes occur, documentation of the following actions or events must be forwarded immediately to ACURO:

- IACUC-approved modifications, suspensions, and triennial reviews of the protocol (All amendments or modifications to previously authorized animal studies must be reviewed and approved by the ACURO prior to initiation.)
- USDA annual program/facility inspection reports
- Reports to OLAW involving this protocol regarding

  a. any serious or continuing noncompliance with the PHS Policy;

  b. any serious deviation from the provisions of the Guide for the Care and Use of Laboratory Animals; or

  c. any suspension of this activity by the IACUC

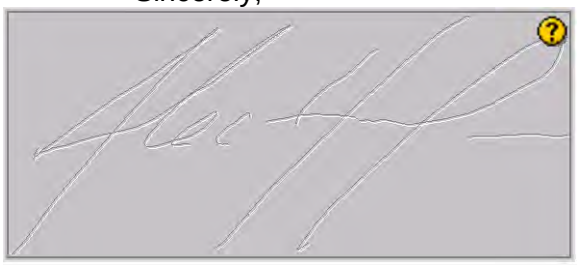
- USDA or OLAW regulatory noncompliance evaluations of the animal facility or program
- AAALAC, International status change (gain or loss of accreditation only)

Throughout the life of the award, the awardee is required to submit animal usage data for inclusion in the DOD Annual Report on Animal Use. Please ensure that the following animal usage information is maintained for submission:

- Species used (must be approved by this office)
- Number of each species used
- USDA Pain Category for all animals used

For further assistance, please contact the Director, Animal Care and Use Review Office at (301) 619-2283, FAX (301) 619-4165, or via e-mail: acuro@amedd.army.mil.

Sincerely,



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